Approaches to Validation
The Science behind Antibody Validation Standards

The following Consensus Building Paper presents key topics described in the following sources: comments from pre-workshop online discussions made on protocols.io; summary data from the validation survey that was completed by Workshop participants and researchers; feedback from the Workshop Steering Committee; and relevant publications. All content not referenced is derived from online discussions. Source material as of August 30, 2016.

The purpose of this paper is to provide a starting off point for the dialogues and consensus-building process at the Antibody Validation: Standards, Policies, and Practices Workshop. The conclusions and preliminary recommendations are not final but are meant to serve as a basis for further discussion.

Antibodies are essential for basic and applied research as among the most widely-used biological reagents, with a more than $1.6 billion dollar market (Baker, 2015). However, only an estimated 30-50% of commercial antibodies demonstrate specificity for their intended targets in the laboratory (Baker, 2015; Bradbury & Pluckthun, 2015), leading to problems with reproducibility, loss of time and money, and hurdles to translation of biomedical research (Baker, 2015; Freedman et al, 2015; Marx, 2013). The inability to replicate published data often can be traced to antibodies that were not properly validated to demonstrate specificity, selectivity, and reproducibility in applications for which they are used (Begley & Ellis, 2012; Bucur et al, 2013; Weller, 2016). Underlying this complex problem is a lack of defined standards on best practices to characterize and validate antibodies (Freedman & Inglese, 2014). Validation standards will enable the use of specific and selective affinity reagents, enhancing the reproducibility of scientific studies. The concepts, perspectives, and preliminary recommendations described herein are derived from the pre-workshop online dialogues, survey, and published literature. The recommendations are intended to promote thoughtful consideration of antibody validation standards for use by producers and researchers, and development of broadly-supported standards that enable the practice of rigorous and reproducible science. This summary paper will serve as a basis for building consensus at the *Antibody Validation: Standards, Policies, and Practices* Workshop.

Proposed Validation Strategies | By validating antibodies, researchers ensure that the reagent has the necessary characteristics to be suitable for its intended experimental use. Antibodies must be shown to bind their intended target (e.g., specificity), to minimally react with other targets (e.g., selectivity), and to consistently and repeatedly work in that manner (e.g., reproducibility) (Bordeaux et al, 2010). The validation strategies, or general approaches for evaluating the specificity and selectivity of an antibody, that have been proposed in online discussions and publications are listed in Figure 1 (Uhlen et al, 2016).

Antibody
Validation
Standards, Policies,
and Practices

GBSI
Global Biological Standards Institute

Genetic Strategies	• Decreased abundance of protein target upon knockdown or knockout • Epitope reactivity correlates with protein expression
Orthogonal/ Complementary Strategies	• Measurement of protein abundance by another method (e.g., an antibody-independent method) that shows concordant results with the antibody tested
Multiple Epitope Strategies	• Comparison of results against a validated antibody that recognizes a distinct epitope of the protein target
Expression Tag Strategies	• Comparison of results against recognition of the tagged protein target
Mass Spectroscopy Strategies	• Determine if an antibody binds the intended protein target by coupling with mass spectroscopy
Cross-application, Predictive Strategies	• Translation of antibody validation data from one method (e.g., protein arrays and western blotting) to inform use in other applications. • In this strategy, the results of one application (e.g., protein arrays) would establish the suitability of an antibody in another application (e.g., immunoprecipitation)
Spatial Localization/ Fractionation Strategies	• Evaluation of antibody signal in a cell-specific and subcellular compartment-specific manner in accordance with existing literature

Figure 1 **Proposed Strategies for Antibody Validation**
Lists antibody validation approaches discussed on protocols.io and in print.

Application-specific Validation | Fit-for-purpose validation is important because experimental context and conditions make developing a single "gold standard" validation strategy that can be used to test antibodies across applications challenging. The primary consequence is the inappropriate assumption that an antibody does or does not work based on the results of the validation approach used. When seeking to develop application-specific validation standards, considerations such as antibody characteristics, experimental conditions, and production-type are important. For example, knowing whether the antibody will be used to recognize native or denatured proteins and whether it was produced using recombinant techniques, created in mouse hybridoma cells, or raised in rabbits inform which validation strategy, controls, and conditions to use. Using mismatched strategies, such as protein arrays which can test an antibody's specificity for recognizing proteins in native conformation, may not reflect antibody specificity for denatured proteins (i.e., in experiments involving fixed or denaturing conditions such as Western blotting, immunohistochemistry, and immunocytochemistry). Based on the survey results and online discussion, certain validation strategies are preferred for some applications but not for others. For example, mass spectroscopy is a highly supported validation strategy for antibodies used in immunoprecipitation, but not for other applications. Similarly, the use of multiple epitope strategies for validating antibodies used in sandwich assays is supported by survey respondents. In addition to these considerations, determining the degree to which antibodies should be tested – including which characteristics (i.e., specificity, selectivity, affinity, etc.) are of greatest relevance, the number of validation strategies used, cost and accessibility of instrumentation, and the need to repeat or reproduce validation data – remain open-ended questions for which additional thought should be given.

Perspectives on Antibody Validation | Summarized in this section are overarching themes for considering the suitability, reliability, and feasibility of the proposed validation strategies. Online discussion highlighted the complexity of antibody validation.

- Biochemical and physical properties of antibodies play important roles in antibody validation.
  - Antigen Form and Accessibility – Analysis of native and non-native antigens requires distinct considerations as the effects of experimental conditions on antigen form and accessibility can vastly affect results (Bordeaux et al, 2010).
  - Biochemistry – The affinity of an antibody against the intended protein target and non-specific targets is dependent on the relative abundance of the protein target in reference samples, and on antibody concentration and incubation times used. Validation strategies that demonstrate antibody specificity and selectivity of endogenous levels of the

protein target are optimal.

- No single validation strategy is sufficient alone. A combination of strategies should be used and ideally, conducted in a "fit-for-purpose" manner (Bordeaux et al, 2010) because experimental and physical conditions vastly influence antibody performance.

- Suitability in one application does not necessarily predict suitability in another. However, predictive strategies, such as the use of high-throughput protein arrays to determine suitability of antibody in another application, may achieve higher efficiency and could serve as an initial validation screen. Other predictive strategies (e.g., Western blotting) are widely accessible and low-cost but may be limited to validation of linear, non-native epitopes.

- The use of novel approaches and technologies, such as mass spectrometry-based methods (Marcon et al, 2015), antibody fingerprinting on peptide and cell arrays, and protein arrays, could improve antibody screening and validation. However, these approaches require further analysis.

  ◦ For example, the benefits and limitations of protein arrays include:

    ▪ Suitability as a validation strategy:

      ▫ Benefits: high-throughput, high efficiency, and high negative predictive value

      ▫ Limitations: lack of thorough validation as a tool, low positive predictive value, limited representation of all possible epitopes found in sample of interest

    ▪ Feasibility as a validation strategy:

      ▫ Benefits: decreasing cost and increasing availability

      ▫ Limitations: low accessibility due to limited use and expertise

- Establishing appropriate negative controls based on cell or tissue-restricted expression is challenging for essential or ubiquitously-expressed genes, especially given the limited understanding of the proteome. The use of calibration sets or reference samples may be advantageous if they can be defined.

- Analysis of antibody selectivity, sensitivity, and specificity against purified protein or in over-expression systems does not demonstrate selectivity. Peptide blocking, although suitable for screening against antibodies present in whole antisera that recognize other protein targets, is not sufficient.

- Validation of antibodies against non-protein epitopes and post-translational modifications requires additional consideration when selecting which validation strategies to use.

In addition to online dialogues, Workshop attendees and researchers were polled on the use of the proposed antibody validation strategies. Based on quantitative analysis of the survey responses (Figure 2A to B, Table 1), several preliminary conclusions on the suitability, practicality, and feasibility of proposed validation strategies can be made.

- Orthogonal/complementary strategies were most commonly identified as essential for antibody validation for all applications. However, the use of transcriptomics and proteomics data that support this strategy must be further explored.

- Genetic and multiple epitope strategies also were frequently considered essential, although to a lesser extent than orthogonal strategies. The use of multiple epitope strategies was emphasized as essential for sandwich assays, reflecting assay design. Feasibility and practicality limit the broad application of these two strategies for all applications.

- Mass spectroscopy strategies were predominantly listed as cost-prohibitive and/or impractical despite being recognized in online dialogues and the literature as having considerable potential as a validation strategy.

- Expression tag strategies were considered beneficial by many respondents but were most frequently identified as

unsuitable for antibody validation.

Figure 2 **Analysis of select antibody validation strategies based on suitability and feasibility and/or practicality.**
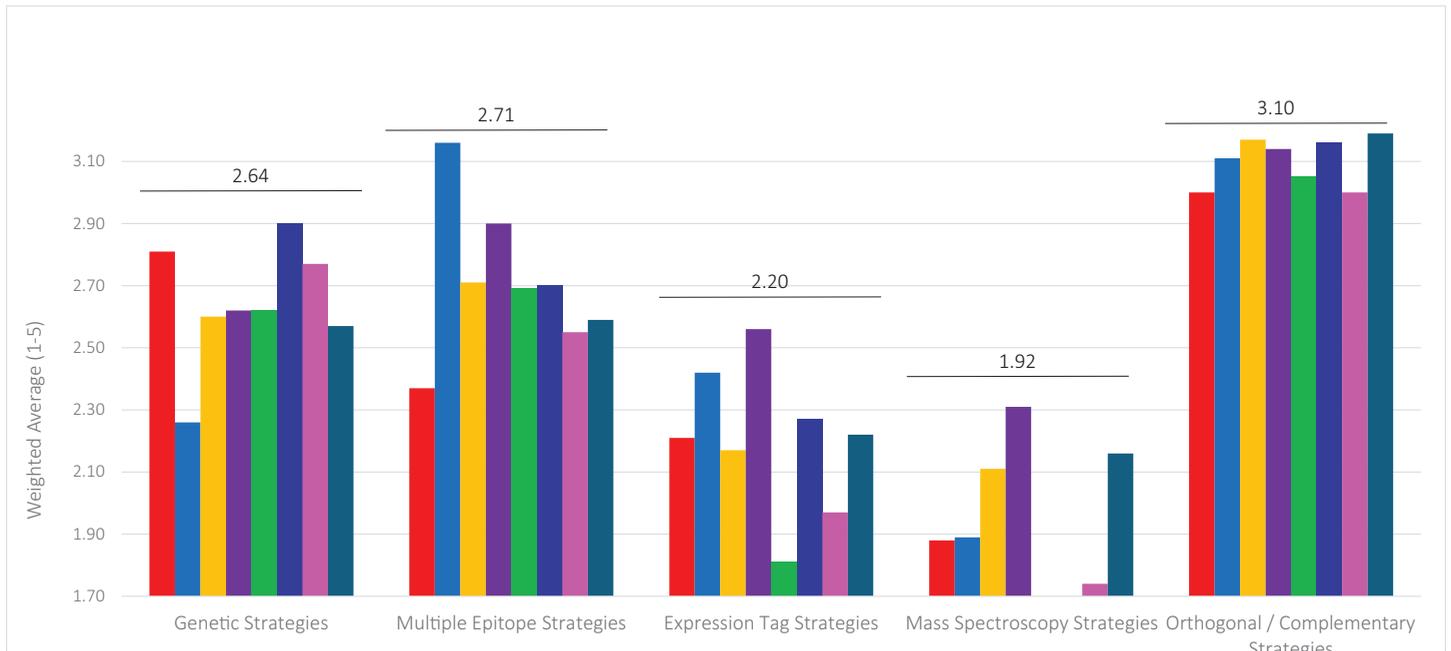

Fig. 2A shows the weighted average (weights shown in parenthesis) of each strategy as determined to be either essential (5), beneficial (4), optional (3), impractical / cost-prohibitive (2), or unsuitable (1) for antibody validation.
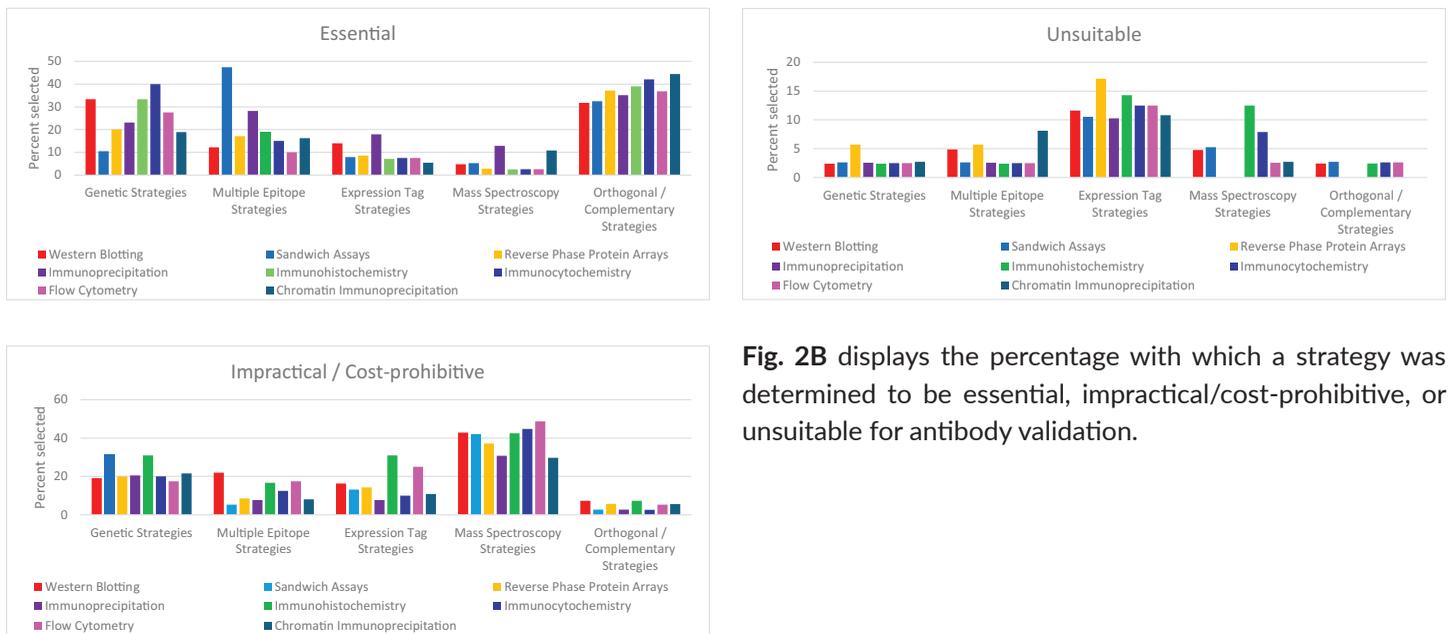

Fig. 2B displays the percentage with which a strategy was determined to be essential, impractical/cost-prohibitive, or unsuitable for antibody validation.

Pillars - Proposed Methods	% "Essential"	Applications							
		Western Blotting	IHC	ICC	Flow Cytometry	Sandwich Assays	IP	ChIP	Reverse Phase Protein Arrays
	Genetic Methods	33	33	40	28	11	23	19	20
	Multiple Epitope Methods	12	19	15	10	47	28	16	17
	Expression Tag Methods	14	7	8	8	8	18	5	9
	Mass Spectroscopy Methods	5	3	3	3	5	13	11	3
	Orthogonal / Complementary Methods	32	39	42	37	32	35	44	37

Table 1 Analysis of select antibody validation strategies based on suitability, feasibility and/or practicality.
Table 1 includes the analysis of survey results. Each validation strategy was determined to be either essential (ranked as 5), beneficial (4), optional (3), impractical/cost-prohibitive (2), or unsuitable (1) for antibody validation in different applications. The coloration shows a heat map [dark blue (5) to white (1)] for the weighted average of each strategy, while the frequency with which an approach was identified as essential in the survey is overlaid.

Formal Validation versus Transparency of Testing in Defined Standards | The validation strategies, as outlined above, represent high-level approaches for testing relevant antibody characteristics. As discussed on the online platform, sharing information on the methods and conditions used during antibody validation is essential for interpreting and verifying validation data. Accordingly, defining standards on the amount and type of information accompanying antibody validation is critical. Potential relevant information, as translated from standards on microarray experiments, is shown in Table 2 (Brazma et al, 2001; Helsby et al, 2013).

Transparency of information for antibody validation	
Antibody Information	[1] Antibody name, [2] Company/supplier name, [3] Host species, [4] Clonality, [5] Catalog and clone number, [6] batch number, if available
Experimental Design	[1] Experimental variables (e.g., validation strategy, time, dose, response to treatment if applicable), [2] Relationship between samples, [3] Quality control, [4] Biological or technical replicates used
Sample Information	[1] Source of sample (e.g., organism, cell type, cell line), [2] Any relevant treatment, growth, or cell/tissue isolation conditions used, [3] sample preparation conditions (e.g., sample lysate buffer, sample concentration, # cells used)
Procedures used (e.g., protocols)	[1] Assay conditions (e.g., blocking conditions, blocking time, epitope retrieval conditions, fixation and permeabilization conditions, antibody incubation times, primary and secondary antibody concentration) [2] Experimental controls (e.g., isotype controls, minus primary antibody conditions, unstained cells)
Quantification and Measurement	[1] Exposure conditions (e.g., time of exposure, relevant settings)

Table 2 Lists relevant types of information needed to assess and reproduce antibody validation results, as adapted from Brazma et al and Helsby et al (Brazma et al, 2001; Helsby et al, 2013.

Recommendations for Discussion | Preliminary recommendations on antibody validation standards based on online dialogues, survey results, and literature are included.

- Standards for antibody validation should be selected based on the results of the online discussions, survey responses, and Workshop dialogue to improve the quality and reproducibility of antibody-based research. These standards should address the selection of appropriate controls, minimum necessary validation strategies, acceptance criteria for antibody validation, and the minimum information needed to evaluate and replicate results.

- Validation of an antibody for use in any application is more informative and reliable if more than one strategy is used. The use of comparative or orthogonal, genetic, or multiple epitope strategies alone is necessary, but not sufficient for antibody validation. The use of cross-application, predictive strategies is promising as a screening approach, but is not sufficient to validate an antibody. In all instances, antibodies must eventually be tested in the intended experimental application (i.e., fit for purpose).

- Selection of controls should be established by the use of appropriate validation strategies and should be informed by the antigen or immunogen, the relative abundance of the protein target, post-translational modifications and protein processing, and expression of related proteins.

- Evaluation of validation strategies should be evidence-based. The use of systematic studies leveraging broad data sets, such as the Human Protein Atlas, or the use of multi-center collaborative studies would enable this. Development of new approaches, technologies, and resources could improve antibody validation further.

- Stakeholders should validate antibodies and communicate the results. Formal validation demonstrates antibody suitability, while information-sharing enables researchers to effectively interpret results. Standards on the minimum level of information needed must be defined, but could be based on existing requirements set by leading life science journals and funders (see Drivers for Adoption paper). Information about the antibody (e.g., unique identifiers like RRIDs, and catalog, clone, and batch numbers), sample materials, sample preparation, and experimental conditions (e.g., antibody concentration, incubation times, and blocking conditions) should be provided.

Proposed Framework for Antibody Validation | The survey and online dialogues provide a preliminary direction for defining a framework for research antibody validation. This framework is intended to inform the development of guidelines for application-specific validation strategies and standards. Given the effects of experimental conditions on antibody performance and need for fit-for-purpose testing, a set of guidelines and validation strategies that are application-specific is necessary. Figure 3 shows a preliminary framework for antibody validation that will be further refined at the Antibody Validation: Standards, Policies, and Practices Workshop.
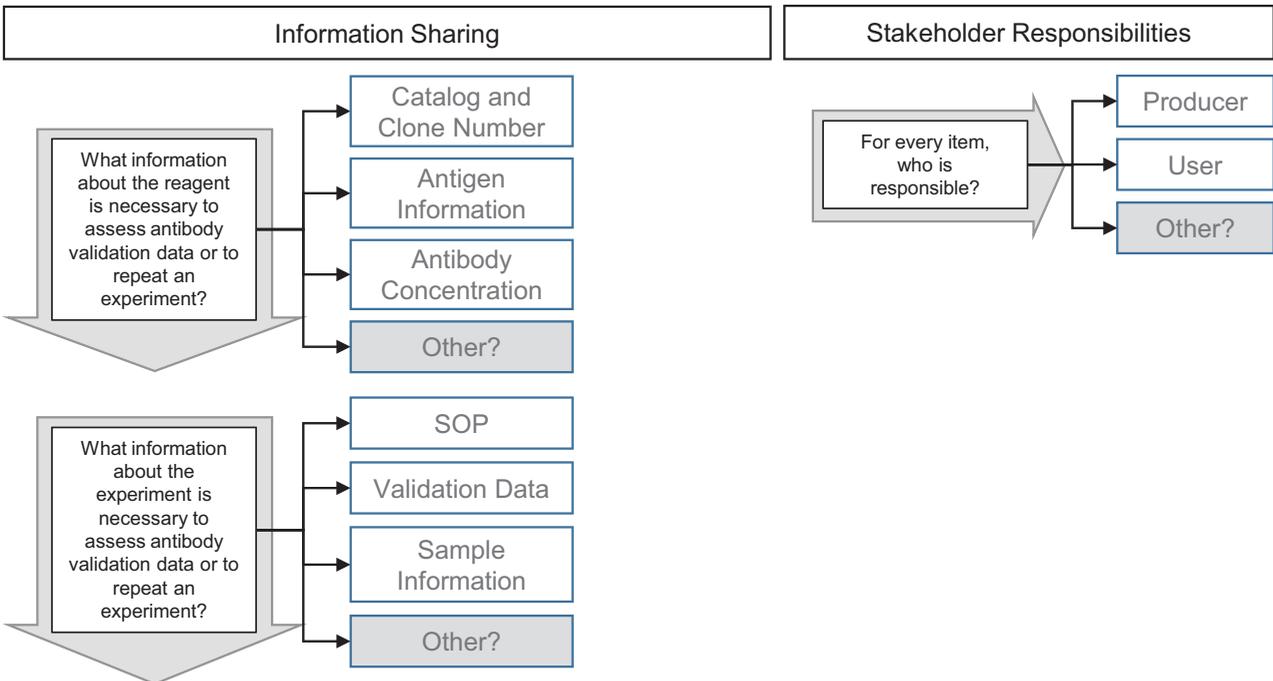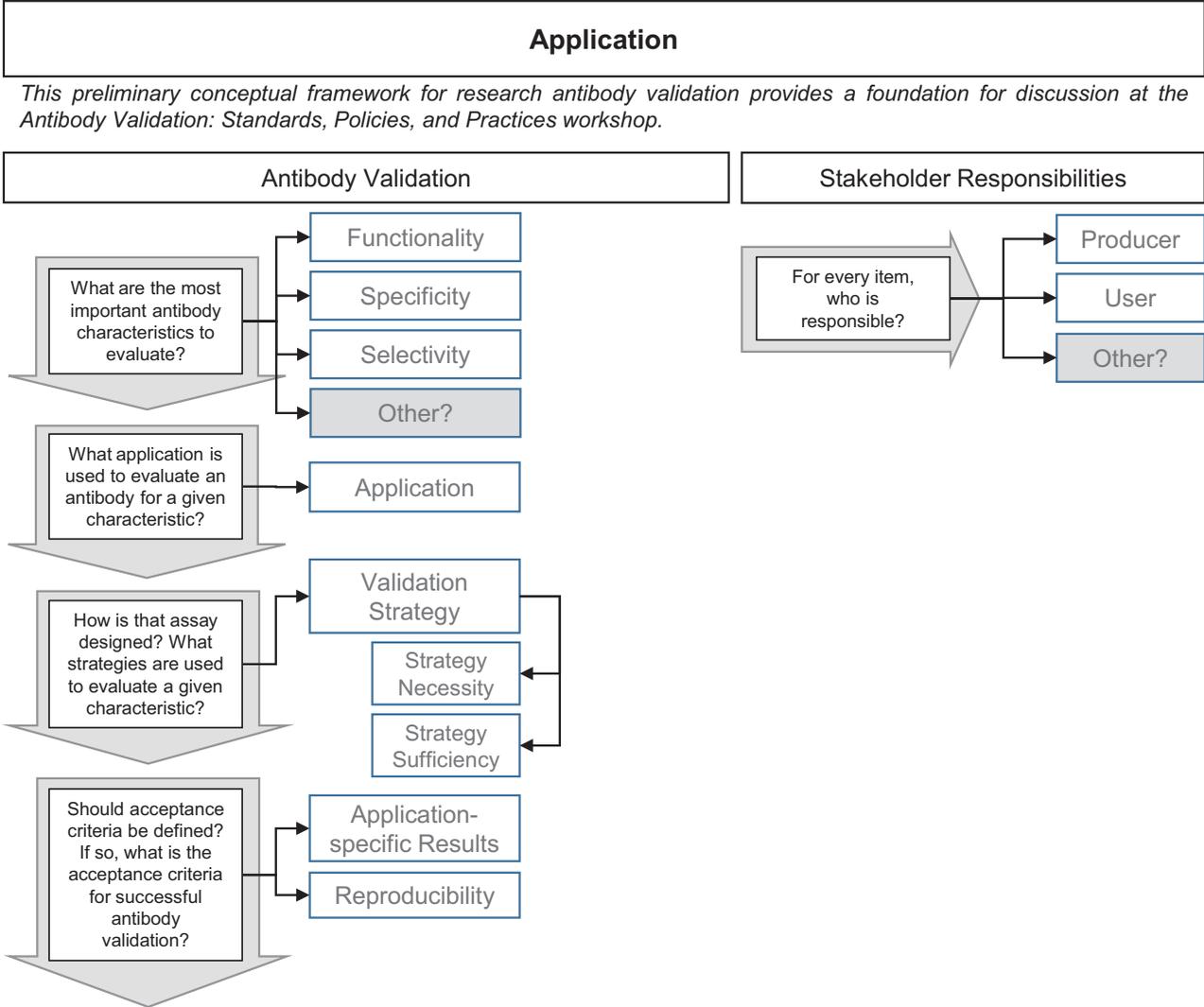
Figure 3 Preliminary framework for antibody validation

Concluding Remarks | Each of the proposed antibody validation strategies has benefits and limitations related to their scientific rigor, applicability across experimental applications and model systems, and ease of use and resource demands. The suitability of a strategy in demonstrating antibody specificity and selectivity is paramount, yet economic considerations (i.e., financial and human resource costs) are important for widespread implementation and adoption. Defined standards should meet the bar of scientific rigor, while also accounting for wide accessibility to the research community. The survey provides a starting point for defining antibody validation standards, but other considerations expressed in the online dialogues require further consideration to drive consensus.

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